

Molecular Mechanics & Force Spectroscopy I

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Extraction of Free Energy Parameters from Dynamic Single-Molecule Force-Extension Measurements

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Dynamic single molecule force spectroscopy (SMFS) provides a powerful approach for probing the free energy landscape that governs how molecules fold into complex 3D architectures, bind to each other, and undergo conformational transitions. In SMFS experiments, a gradually increasing force is applied to a single molecule or complex and the resulting force-extension behavior is recorded until rupture of the system. The analysis of force-extension measurements to recover the intrinsic energy landscape of the system is an outstanding challenge in SMFS. In this talk, I will describe the development of new theoretical models for extracting the height and location of activation energy barriers and their intrinsic transition rates from SMFS measurements [1, 2]. These models improve on the current state-of-the-art by accounting for both the finite stiffness of the pulling device and the non-linear stretching behavior of the molecular handles connecting the device to the system under study. I will end the talk by reporting the results of steered molecular dynamics simulations in which we successfully applied our theoretical models to extract the free energy parameters of representative ligand-receptor unbinding transitions.

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Single Molecule Oxidative Folding

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More than a third of human proteins contain disulfide bonds, yet our understanding of how these are formed *in vivo* has remained rudimentary. Formation of disulfides takes place during oxidative folding and is catalyzed by the enzyme Protein Disulfide Isomerase (PDI). We have developed a single molecule approach to study this process in real-time, using force-clamp AFM. We investigated the oxidative folding of human titin domains, in the presence and absence of oxidase enzymes such as human PDI. Our approach enables, for the first time, independent kinetic measurements of folding and disulfide formation in individual proteins. Strikingly, we discovered a critical period for disulfide formation in the titin I1 domain. After protein folding was completed, disulfide formation could no longer be catalyzed by PDI. Thus, the competing kinetics of PDI-mediated oxidation and protein folding determines the outcome of oxidative folding. The involvement of glutathione in oxidative folding has long been under speculation. We show that glutathionylation can retard the kinetics of protein folding by more than 10-fold, revealing a possible novel role of this ubiquitous small molecule. In protein substrates with more than two cysteines, our data show that correct pairing is achieved through substrate folding rather than through the action of the oxidase enzyme. Therefore, the accuracy of cysteine pairing increases along the folding pathway of the substrate. These unprecedented observations shed new light on how disulfide formation relates to protein folding *in vivo*. Taken together, we are able to construct a complete kinetic model of oxidative folding, incorporating experimentally measured rates for the individual reaction steps. The technique presented here can be used to elucidate the pathway of oxidative folding for a wide variety of proteins, and can also be used to identify mechanisms that trigger misfolding and disease.

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Mechanical Properties of NRR Domain from Human Notch 1 Studied by Single Molecule AFM Force Spectroscopy

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For proteins in living cells forces are present from macroscopic to single molecule levels. Single molecule atomic force microscopy (AFM) in force-extension (FX) and force-clamp (FC) modes has been successfully used to investigate mechanical properties of proteins held under force with ~ 10 pN force sensitivity. FX-AFM measures forces at which proteins (and embedded within them single bonds) undergo conformational transitions. FC-AFM studies yield kinetics of these processes.

Here, we present the results of the FX-AFM experiments on a protein construct comprising the NRR domain from human Notch 1. Notch is a transmembrane cell signaling protein. It is believed that understanding its mechanical properties at the single molecule level will contribute to elucidating Notch's role in processes relevant to embryonic development, tissue homeostasis, and some breast cancers. Our FX-AFM studies and further analysis (namely some maximum likelihood analysis) revealed several conformational transitions possibly related to Notch signaling processes. These results opened a path for further investigations of Notch constructs at various physiologically relevant conditions.

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Force Spectroscopy of E- and N-Cadherin Interactions using Microspheres Arrayed on AFM Cantilevers

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E- and N-cadherins are homophilic adhesion proteins that mediate vital functions like cell-cell contacts, tissue formation, and cell motility. They are also linked to pathological processes like cancer-cell propagation: the increase of cancer-cell motility during the epithelial-mesenchymal transition at the onset of metastasis has been attributed in part to a switch from E- to N-cadherin expression. The dynamic strengths of E- and N-cadherin homophilic (E:E, N:N) and heterophilic (E:N) bonds are thought to play a key role in this process. To redress conflicting literature reports, we here examine the E- and N-cadherin binding strengths using a novel, custom-designed force probe that integrates a horizontal atomic force microscope (AFM) with micropipette manipulation. Unlike past studies, we test E- and N-cadherin proteins from the same (human) species to eliminate uncertainties stemming from interspecies cadherin pairing. Moreover, by assembling arrays of cadherin and control beads onto the same probe cantilevers, we are able to compare the strengths of all combinations of interactions (E:E, N:N, E:N, and controls) more directly than previously possible. Using this unique approach, we establish that E:E cadherin bonds are significantly stronger than N:N cadherin bonds for all tested force-loading rates, with heterophilic E:N cadherin bonds possessing an intermediate strength.

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High Permeability Silicone-Based Magnetic Microspheres for Microscale Force Spectroscopy

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A wide variety of magnetic microspheres are currently available for applications such as cell sorting, chemical separations, or microscale force spectroscopy. The smallest spheres (10 nm - 500 nm) generally consist of solid iron-oxide nanoparticles synthesized via the coprecipitation of iron salts, while many larger products (2 μ m - 100 μ m) consist of polymer microspheres which are subsequently saturated with magnetic nanoparticles. However, the latter process may result in incomplete saturation and therefore diminishing magnetic concentration as sphere diameter increases. In addition, a lack of confluence between these two methods of production has resulted in a dearth of products in the 0.5 - 2 μ m range. We present here a bottom-up approach to magnetic microsphere fabrication which allows us to scale the diameter of our microspheres between 0.2 - 100 microns while maintaining a constant magnetic content throughout. In this approach, we use a novel magnetic silicone consisting of magnetite nanoparticles complexed with a monolayer of poly(dimethyl siloxane-co-aminopropylmethyl siloxane). The magnetic content of the composite may be tuned from 0 - 50% wt. without nanoparticle aggregation, resulting in a highly magnetic silicone fluid which is homogenous at scales below 100 nm. We demonstrate the production of microspheres of this material by an emulsion process in which microsphere diameter may be tuned by careful selection of surfactant type and concentration. Finally, we show that the resulting spheres compare favorably with leading commercial competitors in terms of magnetic force application.

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Rupture Force of Single Small Drug Molecule Binding a Split Aptamer

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Aptamers are specific oligonucleotides (DNA or RNA) which bind small inorganic or organic molecules, large proteins or cells. In particular, the high affinity of aptamers is expected to lead to a new class of therapeutic reagents. Thus the detection and characterization of binding strength of small molecules is important for drug and medical research. Atomic force spectroscopy (AFS) with a force resolution in the piconewton range is a valuable tool for studying interactions on a single molecular level. The detection of very small target molecules less than 500 Dalton is characterized by only a few hydrogen interactions between the aptamer and the target molecules. Thus tiny rupture forces well below 100 pN are predictable. For AFS the target molecules as well as the aptamer probes are typically immobilized on the AFM-tip or sample surface, respectively. This concept suffers from the need to chemically manipulate or label the target analyte by binding sites that interact with surfaces. We solved this problem by using a split aptamer probe. Both components of the aptamer were immobilized on the SFM-tip and sample surface, respectively. During the AFS experiment the split aptamers form defined binding pockets for the free analyte. The concept of using a split aptamer allowed the detection of the